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First Description of an RND-Type Multidrug Efflux Pump in *Achromobacter xylosoxidans*, AxyABM▽

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Achromobacter xylosoxidans **is an emerging pathogen in cystic fibrosis patients. The multidrug resistance of these bacteria remains poorly understood. We have characterized in a clinical strain the first resistance-nodulation-cell division (RND)-type multidrug efflux pump in this species: AxyABM. The inactivation of the transporter component *axyB* gene led to decreased MICs of cephalosporins (except cefepime), aztreonam, nalidixic acid, fluoroquinolones, and chloramphenicol.**

Achromobacter xylosoxidans is a nonfermentative Gram-negative bacillus considered to be an opportunistic agent (1, 25). It is an emerging pathogen in cystic fibrosis (CF) (6, 20). Currently, 120 patients are followed at our CF center, of whom 24 (20%) have been colonized at least once by *A. xylosoxidans*. It has recently been suggested that *A. xylosoxidans* might be responsible for a high inflammatory response and therefore might be involved in the decline in lung function (9). This microorganism exhibits innate antibiotic multiresistance, including resistance to cephalosporins (except ceftazidime), aztreonam, and aminoglycosides (1, 2, 8, 22). There are only a few reports that describe resistance mechanisms in this species. To date, only the constitutive oxacillinase OXA-114 (7) and a couple of acquired β-lactamases belonging to VEB, IMP, and VIM families have been described (11, 15, 21, 23, 24). Nevertheless the mechanisms of intrinsic resistance of *A. xylosoxidans* to cephalosporins, aztreonam, and aminoglycosides remain unknown.

Resistance-nodulation-cell division (RND)-type efflux pumps are widely present among nonfermentative Gram-negative bacilli (3–5, 10, 14, 17, 19). In a preliminary experiment on *A. xylosoxidans*, we investigated the effect of reserpine, an efflux pump inhibitor, on MIC values of various antibiotics commonly used to treat CF patients. We observed an increased activity of drugs from different families such as β-lactams, fluoroquinolones, and tetracyclines. This prompted us to investigate the presence of an efflux mechanism in this species.

For this purpose, we chose the clinical strain AXX-A, isolated in our laboratory. This strain harbors a wild-type antibiotic resistance phenotype (Table 1): it is susceptible to ticarcillin, ceftazidime, carbapenems, and ciprofloxacin but resistant to cephalothin, cefoxitin, cefotaxime, cefepime, aztreonam, nalidixic acid, and aminoglycosides. The strain was identified by using the API20NE system (bioMérieux, Marcy l'Etoile, France) and confirmed by 16S rRNA gene sequencing.

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We designed multiple sets of primers in conserved regions of RND efflux genes from *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Burkholderia cenocepacia*. With the primers MexB2-F and MexB2-R (Table 2) we amplified in AXX-A a 1,097-bp sequence sharing 88% similarity with the *acrB* gene from *Bordetella bronchiseptica*. We then determined the complete sequence of a putative RND efflux operon by using the two-step gene walking method (16). This operon is composed of three putative open reading frames (ORFs) encoding a membrane fusion protein (designated AxyA), a RND transporter protein (AxyB), and an outer membrane protein (AxyM). Upstream and in an inverted orientation we detected an ORF encoding a transcriptional regulator belonging to the LysR family (AxyR) without any homology with MexR.

The standard protein BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to detect protein sequence similarities. The gene products AxyA, AxyB, and AxyM showed very strong homologies with putative RND-type efflux proteins from *A. xylosoxidans* A8, *Achromobacter piechaudii* ATCC 43553, and *Bordetella bronchiseptica* RB50 (Fig. 1). The putative efflux systems have not been studied in these species. Nevertheless, AxyB shares 72% identity with the *P. aeruginosa* MexB protein, which is the RND transporter component of the well-characterized MexAB-OprM efflux system (18). We sequenced the *axyABM* operon in both AXX-A and another clinical strain (AXX-C) entirely. While AXX-A and AXX-C AxyABMs share 99% similarity, the percentage of identity was surprisingly lower (90 to 94%) for AxyABM from the environmental strain *A. xylosoxidans* A8.

We studied the role of AxyABM in the innate antibiotic resistance of *A. xylosoxidans* by inactivating *axyB* in AXX-A. The suicide plasmid pINAP1, derived from vector pUC18, was constructed in *Escherichia coli* DH5α cells by cloning a 977-bp EcoRI-HindIII PCR fragment internal to the *axyB* gene (nucleotide positions 3847 to 4823 in *axyABM*) obtained with primers INA-axyB-2F and INA-axyB-2R. Then pINAP1 was introduced into AXX-A by electroporation. The recombinant AXX-A (*axyB*::Tic), named AXX-A-ΔP, was selected on Mueller-Hinton agar plates containing 50 μg/ml of ticarcillin. The *axyB* disruption by pINAP1 integration was confirmed by

TABLE 1. MICs of 24 antibiotics for AXX-A and AXX-A-ΔP (*axyB*::Tic)

Antibiotic	MIC (μg/ml) for:	
	AXX-A	AXX-A-ΔP
Ticarcillin	0.25	>256
Ticarcillin-clavulanic acid	0.5	0.5
Cephalothin	24	12
Cefuroxime	>256	>256
Cefotetan	>256	32
Cefoxitin	>256	128
Ceftriaxone	≥256	12
Cefotaxime	>256	12
Ceftazidime	4	1.5
Cefixime	>256	32
Cefepime	16	16
Aztreonam	>256	16
Imipenem	1	1
Meropenem	0.094	0.094
Nalidixic acid	24	6
Norfloxacin	8	4
Ofloxacin	2	1
Levofloxacin	0.75	0.38
Ciprofloxacin	0.75	0.5
Tobramycin	16	16
Amikacin	≥256	≥256
Tigecycline	4	4
Colistin	4	4
Chloramphenicol	12	6

PCRs (two sets of primers, AL-BAP1-F1/M14 and M14R/AL-BAP1-R1). The absence of *axyB* expression in AXX-A-ΔP was demonstrated by reverse transcription-PCR with primers RT-INA-R0 and RT-INA-F1 (AXX-A was used as a positive control). We determined the MICs of a panel of antibiotics for AXX-A and AXX-A-ΔP by using the Etest method (bioMérieux, Marcy l'Etoile, France). The MICs of most of the cephalosporins and aztreonam drastically decreased as a result of the *axyB* inactivation (by more than 20-fold for ceftriaxone and cefotaxime) (Table 1). The activity of nalidixic acid, fluoroquinolones, and chloramphenicol was also enhanced in AXX-A-ΔP. Thus, the inactivation of *axyB* in AXX-A led to increased susceptibility to several antibiotics belonging to different families. However, no variation was noticed for imipenem, meropenem, colistin, tigecycline, and aminoglycosides. Therefore, AxyABM is not involved in the innate resistance of *A. xylosoxidans* to aminoglycosides. Spontaneous revertants were obtained by cultivating AXX-A-ΔP without ticarcillin. The loss of the inserted pINAP1, confirmed by PCRs, led to phenotypic reversion.

TABLE 2. Primers used in the study

Primer	Nucleotide sequence (5′–3′)
MexB2-F	AACGTGCAGATTTCCTCCGG
MexB2-R	TGTACCTGGGCGAACAGTAC
INA-axyB-2F	GGGGAATTCTCTATCGCCAGTTCTCCATCA[a]
INA-axyB-2R	GGGAAGCTTGCGCACGTACCATTTGTCCA[b]
AL-BAP1-F1	TGTACCTGTTCCTGCAGAAC
M14F[c]	CCAGGGTTTTCCCAGTCACGA
M14R[c]	GCGGATAACAATTTCACACAGGA
AL-BAP1-R1	AAGTACACGTCGTTGGACAG
RT-INA-R0	ACCATGTCGCCATCCTTGTT
RT-INA-F1	GTGTTCATCCCGATGGCGTT

[a] The EcoRI restriction site introduced into the primer is underlined.
[b] The HindIII restriction site introduced into the primer is underlined.
[c] Primer designed in pINAP1.

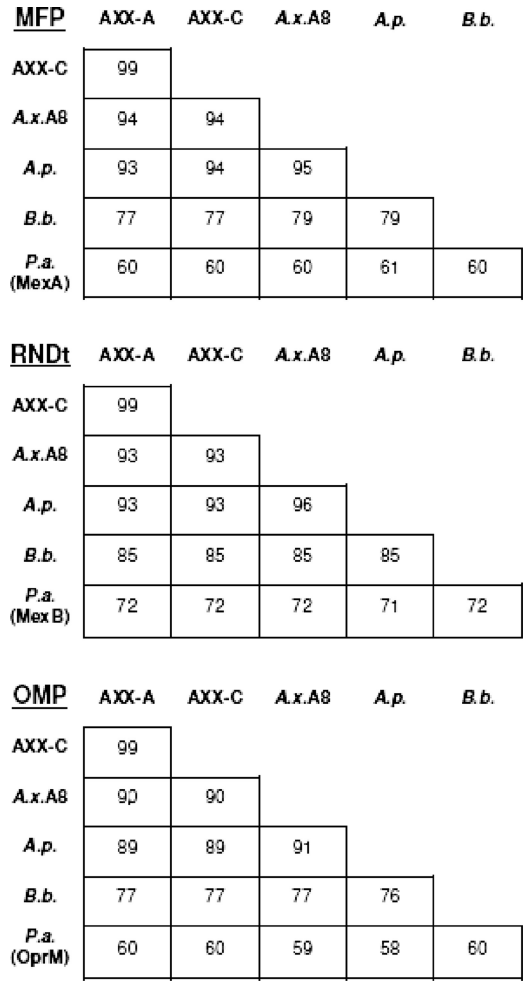

FIG. 1. Amino acid sequence similarities (%). MFP, membrane fusion protein (AxyA and homologues); RNDt, RND transporter (AxyB and homologues); OMP, outer membrane protein (AxyM and homologues); *A.x.* A8, *A. xylosoxidans* A8; *A.p.*, *Achromobacter piechaudii* ATCC 43553; *B.b.*, *Bordetella bronchiseptica* RB50; *P.a.*, *Pseudomonas aeruginosa* PAO1.

This is the first description of an antibiotic resistance mechanism by efflux within the genus *Achromobacter*. Antibiotics commonly used for the management of lung infections in CF patients, like ceftazidime and ciprofloxacin, are substrates of this efflux system. AxyABM shares some properties with MexAB-OprM from *P. aeruginosa* since it exports β-lactams (except imipenem), fluoroquinolones, and chloramphenicol but not aminoglycosides (12, 13, 18). However, unlike MexAB-OprM, AxyABM exports neither cefepime nor meropenem. From our preliminary study, we noticed a decrease in tigecycline MIC in the presence of reserpine for the AXX-A strain. These data strongly suggest that tigecycline is a substrate for an efflux system. The MIC of tigecycline was not changed following *axyB* inactivation in AXX-A; tigecycline is therefore not a substrate for AxyABM. It is likely that other efflux systems take part in the antibiotic resistance of *A. xylosoxidans*.

In conclusion, we have shown that the AxyABM efflux system is one of the mechanisms involved in the innate multidrug resistance of *A. xylosoxidans*. Furthermore, it can extrude antibiotics widely used to treat CF patients. Therefore, its role in acquired antibiotic resistance and its regulation mechanisms remain to be explored.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned accession number JF514544 in the GenBank database.

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